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PARAFFIN SECTION THICKNESS— A DIRECT METHOD OF MEASUREMENT

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ABSTRACT.—**Paraffin section thickness may be directly measured by re-embedding the sections under consideration, cutting them again at right angles to the original plane of sectioning, and taking direct measurements with a filar micrometer after staining and mounting. Conditions and materials with which relatively undistorted 3 and 5 μ sections were secured include (a) a hand-honed knife with a 23° facet bevel, set at a clearance angle of 7°, and (b) a hard paraffin (56–58°) embedding medium, preferably with 5% beeswax and 5% bayberry wax added. By taking direct measurements, it was found that bull testis tissue cut at a microtome setting of 10 μ averaged 10.82 μ in thickness. Settings of 5 μ and 3 μ resulted in sections averaging 5.25 and 3.31 μ in thickness respectively. Stages in sporogenesis of *Onoclea sensibilis*, Lewitsky fixed, were examined after sectioning at settings of 10, 5, and 3 μ to determine necessity for thin sections. For this material, it was indicated that mitochondrial preparations as thick as 10 μ were worthless, regardless of good fixation and proper staining. Three-micron sections give the best results.**

INTRODUCTION

The question of the relation of actual thickness of paraffin sections to the microtome setting has been approached in a number of different ways. One familiar method is to determine the depth of focus of the section by means of the calibrated fine adjustment of the microscope. John (1929a) found this to be unreliable, and showed that in some cases the error might exceed the thickness of the section itself. Pusey (1939) describes the method of marking off 1 cm. on the paraffin block and then dividing 10,000 by the number of sections cut from this length, the resulting figure being the average thickness in micra of the sections cut at a given setting. This method might be satisfactory in cases where tissue compression is negligible, but it does not take into consideration the excess thick-

ness which might be met in extremely thin sections. John (1929b) measured section thickness using an optimeter, and Richards (1942) applied the principles of interferometry to section thickness measurements. The latter method requires the use of a special slide and cover glass, and illumination by sodium light. Both these methods involve equipment not available to the average laboratory.

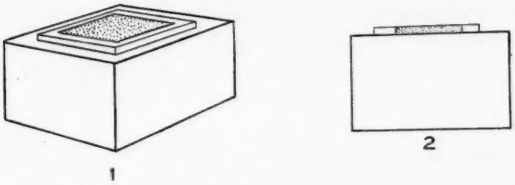
Dempster (1943) computed section thickness on the basis of percentage compression indicated by the ratio of floated-out section length to original block measurement. Using a 27° bevel angle knife at a 1° clearance angle, the thickness of sections cut at a setting of 10 μ, showing a compression of 27.5%, was computed to be 13.8 μ. With smaller microtome settings, increasing compression and excess thickness were found. At 5 μ setting, the compression was 57.8%, and the computed thickness 11.9μ, and at 2.5 μ setting, compression was 83.9% and the computed thickness 15.5 μ. The embedding medium was 52° "Bioloid" paraffin.

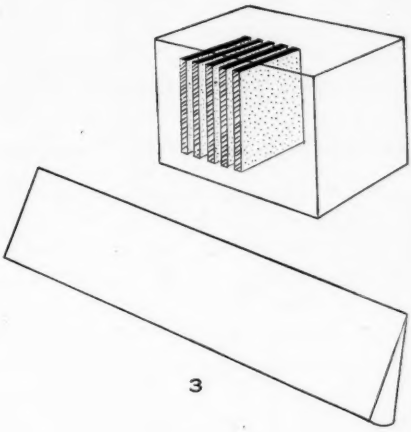
Having been at work for some time on a problem in plant cytology (i.e. the study of mitochondria and plastid primordia) requiring sections of 3 and 4 μ in thickness, it was surprising to the author to learn that on the basis of the last mentioned paper, laboriously prepared 3 μ sections might be anywhere between 11.9 and 15.5 μ in thickness. The literature concerning cytoplasmic inclusions contains numerous references to 4, 3, and even 2 μ sections. The figures of Senjaninova (1927) and Lewitsky (1925) show no distortion of cells, in spite of the fact that sections were cut 5 μ and below. The standard texts in microtechnic (Guyer 1917, Johansen 1940, Lee 1937, Mallory 1938, McClung 1937) all recommend sections 5 μ or below for mitochondrial preparations, and it was on the basis of these, and related papers, that 4 and 3 μ sections were used.

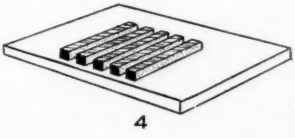
In view of the conflicting material in regard to distortion and excess thickness, a method was devised to determine directly the actual thickness of sections cut at 10, 5, and 3 μ settings, under conditions of sectioning identical to those of the cytological study mentioned above. Also considered was the problem of the necessity of cutting sections as thin as 3 and 4 μ for the study of the cytoplasmic inclusions under consideration. The relative tissue distortion appearing in sections cut at 10, 5, and 3 μ settings was also investigated.

Materials and Methods—General

The embedding medium consisted of 90 parts by weight of 56–58° "Bioloid" paraffin, 5 parts Eimer and Amend bayberry wax and 5







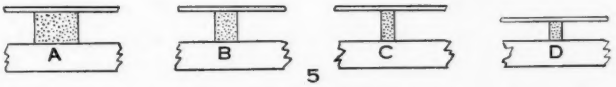


Figure 1.—Diagram illustrating method of re-embedding tissue sections for resectioning. The paraffin section is placed on the surface of a trimmed paraffin block. When the heated tip of a small screw driver is brought over the section, the fusion of the paraffin of the section and that of the block embeds the tissue.

Figure 2.—Diagram of side view of figure 1.

Figure 3.—Diagram illustrating orientation for cutting of the re-embedded tissue sections.

Figure 4.—Diagram of a section cut from the block in figure 3.

Figure 5.— Diagrams illustrating slide, tissue and coverglass relations of the finished slides. (A) Original 10 μ section resectioned at 7 μ; (B) original 5 μ section resectioned at 7 μ; (C) original 3 μ section resectioned at 7 μ; (D) original 3 μ section resectioned at 4 μ.

parts Eimer and Amend yellow beeswax (Rugh, 1941). A Spencer rotary microtome, No. 820, was used. The knife had a 23° facet bevel, and had been hand-honed on a Droescher blue-green hone and finished on a Belgian yellow hone. It was set at a clearance angle of 7°.

Bouin-fixed bull testis tissue was used in the thickness determination and was also examined for cellular distortion at low microtome settings. Fertile frond segments of *Onoclea sensibilis*, Lewitsky-fixed, were used to determine the necessity of sections below 5 μ for accurate study of cytoplasmic inclusions. All the material was stained by the iron-hematoxylin method.

Determination of Section Thickness

To prepare sections for thickness measurements, they were cut at the desired microtome setting, re-embedded, then cut again so that after mounting and staining, a series of cross sections (Fig. 4) of the original sections would be ready for direct measurement with a filar micrometer.

Ribbons were cut from the same block of testis material at settings of 10, 5, and 3 μ. Re-embedding of the individual sections for re-sectioning was accomplished as follows: A block of the embedding medium was trimmed to a size slightly larger on its top surface than the sections of the ribbon being considered. A section from the ribbon was then cut off, and carefully centered on the top of the trimmed block (Fig. 1, 2). The tip of a small screw driver was heated and carefully brought to a point over the section. As this was slowly brought nearer the transferred section, the paraffin of the section and that of the block surface melted. When the heat was removed, the solidification of the medium embedded the tissue section in the top of the block. The procedure was repeated until a compact stack of sections was embedded. When sufficient sections had been transferred, a layer of the medium was added on top of them, and the block was then ready for trimming.

The block was oriented for cutting so that there would be no possibility of compression in the direction of section thickness measurement (Fig. 3). The sections were then cut and mounted in the usual manner. Following removal of the paraffin prior to staining, the slides were coated with thin celloidin by dipping them in 0.5%–0.25% celloidin in equal parts of absolute alcohol and ether. Hardening the celloidin was accomplished by partially air-drying the slides prior to placing them in 95% alcohol. This procedure prevents displacement or washing-off of tissue sections.

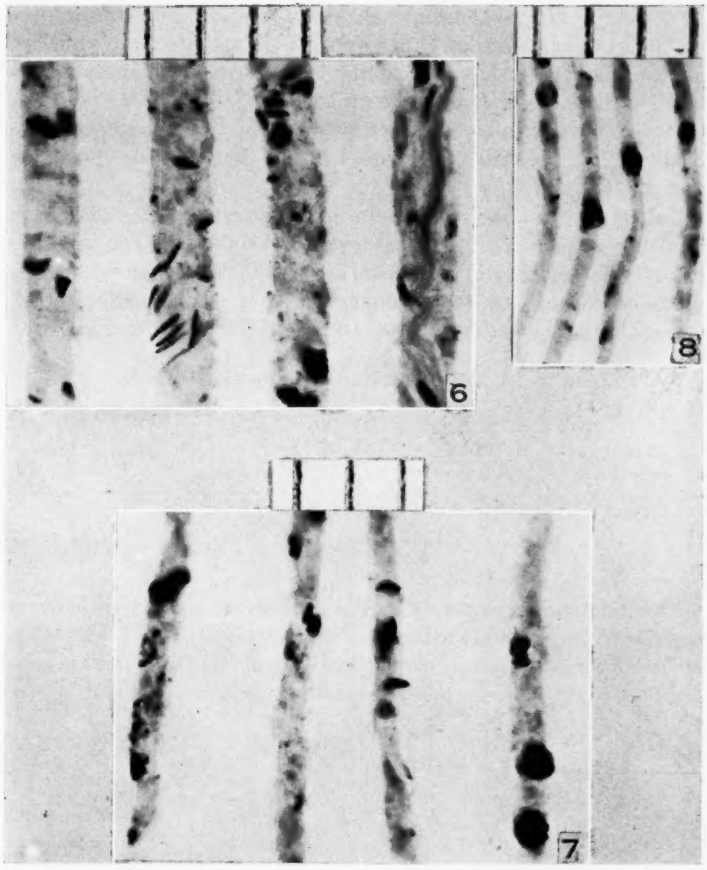


Figure 6.—Cross sections of 10 μ bull testis sections.
Figure 7.—Cross sections of 5 μ bull testis sections.
Figure 8.—Cross sections of 3 μ bull testis sections.
All figures 850×, Bouin fixation, iron hematoxylin. The stage micrometer spaces above each group of sections are equivalent to 10 μ each, and were photographed at same magnification.

For the sections cut at 10 and 5 μ settings, a second cutting at 7 μ proved satisfactory, the strips on the slide presumably being 10 μ wide by 7 μ high in the first case, and 5 μ wide by 7 μ high in the second (Fig. 5a, b). With the 3 μ sections, however, the 3 μ by 7 μ strips (Fig. 5c) did not all remain upright, and by the time the slides were stained and the cover glasses mounted, the majority of them had fallen over so that the 3 μ edge was no longer uppermost. Changing the second cutting settings of the 3 μ sections to 4 μ resulted in more satisfactory slides, and measurements were readily made on these, the strips 4 μ high by 3 μ wide remaining upright (Fig. 5d).

Table 1 summarizes the results of 30 determinations of section thickness at each of the original microtome settings. The measurements were taken with a Bausch and Lomb filar micrometer calibrated by means of a stage micrometer to use with a 44× objective. One dial interval was determined to be equivalent to 0.211 μ.

TABLE 1. THE RELATION OF AVERAGE THICKNESS OF SECTIONS TO MICROTOME SETTING.

Microtome Setting	Average No. Dial Intervals	Average Thickness	Average Excess Thickness
10 μ	51.3	10.82 μ	0.82 μ
5	24.9	5.25	0.25
3	16.53	3.31	0.31

The photomicrographs (Fig. 6, 7, 8) show characteristic cross sections of the original sections cut at the three settings used. Ten-micron spaces of a stage micrometer photographed at the same magnification are shown above each group of sections.

EXPLANATION OF PLATE 3

Figure 9.—Portion of a semeniferous tubule of bull testis. Bouin fixation, iron hematoxylin. Thickness 3 μ.

Figure 10.—Same as figure 9. Thickness 5 μ.

Figure 11.—Same as figure 9. Thickness 10 μ.

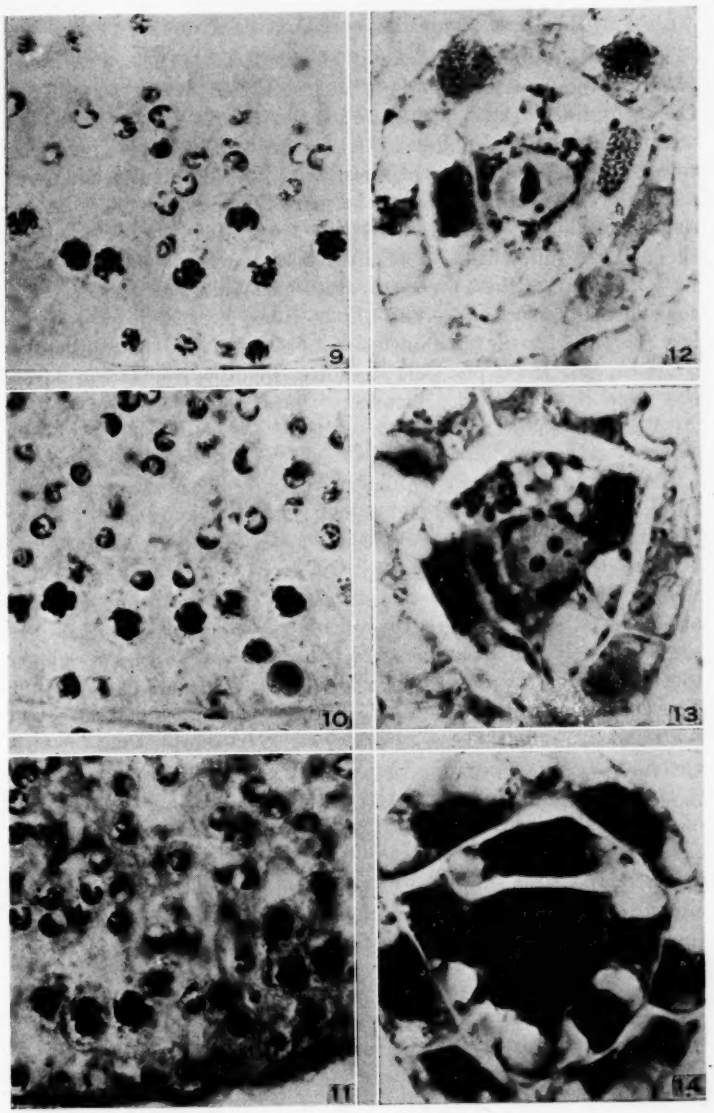
Figures 9, 10, and 11 are from the same block of tissue, and from the same slide. 500×.

Figure 12.—Young sporangium of *Onoclea sensibilis* showing stained inclusions of central sporogenous cell and tapetum. Lewitsky fixation, iron hematoxylin. Thickness 3 μ.

Figure 13.—Same as figure 12. Thickness 5 μ.

Figure 14.—Same as figure 12. Thickness 10 μ. Note poor differentiation of cytoplasmic inclusions which show clearly in figures 12 and 13.

Figures 12, 13, and 14 are from the same block of tissue and from the same slide. 850×.


9
12
10
13
11
14

The Necessity for Thin Sections

A block containing a segment of a fertile frond of *Onoclea sensibilis*, fixed by the Lewitsky method, was sectioned at 10, 5, and 3 μ. On each slide, a strip of 10 sections of each thickness of ribbon was mounted. The different thicknesses of tissue on a given slide were thus subjected to identical staining and differentiation times, and an accurate comparison of the three thicknesses made. Microscopic examination indicated that the 3 μ sections (Fig. 12) showed the clearest differentiation of the stained cytoplasmic inclusions. At 5 μ, (Fig. 13) these were still distinguishable, but not as clearly so as in the 3 μ sections. The 10 μ sections (Fig. 14) proved worthless for the study of the inclusions preserved by this method of fixation. All three photomicrographs (Fig. 12, 13, 14) are from the same slide. Identical exposure times were used.

Tissue Distortion

Bouin-fixed bull testis tissue was cut at microtome settings of 10, 5, and 3 μ. The different thicknesses of ribbon were cut from the same block, which had been trimmed so that its thickness was uniform throughout its length. On each slide were mounted 3 strips of 10 sections from each thickness of ribbon. Sections cut at all three settings were thus floated out, affixed, and stained simultaneously. Measurements on the finished slides showed the total length of the 90-section ribbon cut at 10 μ to be 403 mm. The 90-section ribbon cut at 5 μ was 372.5 mm., and that cut at 3 μ was 360.5 mm. The 5 μ ribbon was 7.5% shorter than the 10 μ one, and the 3 μ ribbon was 10.5% shorter than the 10 μ one.

The small amounts of compression shown by the measurements of total ribbon length do not appear to be significant when the tissue is examined microscopically. The nuclei of parallel stages in spermatogenesis shown (Fig. 9, 10, 11) appear to be of the same size in spite of the difference in the microtome settings at which they were cut. All three photomicrographs are from the same slide.

Discussion

The results indicate that under the sectioning conditions followed, relatively undistorted paraffin sections are possible with settings as low as 3 μ. While the nature of the tissue and the embedding medium undoubtedly have some bearing on the amount of distortion, it is believed that the acuteness of the bevel angle of the knife is the critical factor in securing undistorted thin sections. A closely graded series of knives with various bevel angles was not available and no extensive work was done to check this belief. However,

an earlier attempt to use a factory machine-honed knife with a 32° bevel resulted in hopelessly mangled and distorted tissue whenever the microtome setting was lower than 8 μ. Rehoning this knife at the same bevel on blue-green and then Belgian yellow hones produced no improvement in its cutting quality. Using the regular carrier on the Spencer 820 rotary microtome, the maximum clearance angle obtainable with this knife was 2.5°. It is possible that the use of a special carrier permitting a greater tilt might make for better cutting. This problem also requires extensive investigation which was not possible at this time.

The use of hard (56–58°) paraffin has proved to be most satisfactory when thin sections are needed. Previous work when pure paraffin of this melting point was used, indicated that with the same knife and the same clearance angle, 5 and 3 μ sections were not appreciably distorted. The addition of beeswax and bayberry wax improves the medium considerably, and ribbons cut at 3 and 5 μ retain their firmness even in hot weather.

On the basis of direct measurements, it appears that the excess thickness resulting from paraffin compression is nowhere near the amount indicated by indirect computations based upon abnormally compressed sections. At the three settings checked (10, 5, 3 μ), the average excess thickness was in all cases under 1 μ. Under similar conditions of sectioning, with similar tissue, the sections should never be more than 1 μ thicker than the microtome setting, at least when cutting 3 μ or above.

The necessity for thin sections in mitochondrial preparations is clearly shown by the failure of these elements to be sharply delineated in 10 μ sections subjected to identical staining and differentiation as sections of 5 and 3 μ. Five-micron sections in some cases appear satisfactory, but the clear definition of these bodies in 3 μ sections is not equaled in cells cut thicker. The trouble involved in securing a hand-honed knife of 23° bevel repays well in the undistorted thin sections produced.

Summary

1. A satisfactory method of direct determination of paraffin section thickness has been described.

2. Conditions and materials with which relatively undistorted 3 and 5 μ sections with excess thickness under 1 μ were secured include (a) a hand-honed knife with a 23° facet bevel, set at a clearance angle of 7°, and (b) a hard paraffin (56–58°) embedding medium, preferably with 5% beeswax and 5% bayberry wax added.

3. For material of a similar nature to that used in this study, mitochondrial preparations of 10 μ in thickness are worthless, regardless of good fixation and proper staining. The 3 μ sections give best results.

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FLUORESCENCE MICROSCOPY APPLIED TO ENTOMOLOGY AND ALLIED FIELDS

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ABSTRACT.—**Fluorescence is a phenomenon observable in many substances including a wide range of biological constituents. By use of ultraviolet illumination and the proper fluorescent dyes, when needed, many details of structure and physiological differentiation are made apparent which by illumination with visible light are obscure.**

The fluorescence microscope is a valuable adjunct to the study of fluorescence in biological materials. This instrument is discussed from a practical standpoint. The simplifications in the instrument which do not impair its efficiency are indicated.

The use of fluorochromes is discussed and a list of the most important of these is given. Important technics with the fluorescence microscope, including intravital microscopy, fluorescent photomicrography, and microspectroscopy, are described.

INTRODUCTION

Fluorescence is a phenomenon observable in many substances which, when exposed to ultraviolet irradiation, absorb energy and emit this in the form of light of a longer wave length than the activating rays. This response was set forth as a physical law by Stokes. From statements accredited to Helmholtz (1874) it is to be expected that the image of a substance will be better differentiated if the microscopic object itself were to emit the illuminating rays. The fluorescence microscope fulfills this condition, and, in addition, makes it possible to estimate qualitatively the constituents of fluorescing materials and the physiological state of living systems. The importance of fluorescence in biological material was recognized by Kohler (1904b), and during the period which followed, the valuable technics of fluorescence microscopy have been developed.

The use of the fluorescence microscope has brought about many important discoveries in the fields of applied biology. A comprehensive listing of these up to 1939 can be found in the review paper of Ellinger (1940b).

Since 1939 papers have appeared describing many phases of

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research with fluorescence technics. The distribution of vitamin A in various tissues has been described by Popper (1941), and by Cornbleet and Popper (1941); and Popper and Greenberg (1941) have reported the differentiation of vitamins A and A_2 in the livers of fish by this technic.

Investigations with the use of fluorescein in intravital studies have been advanced by Evans and Singer (1941) with their studies of the function of the eye.

The use of fluorescence technics for the demonstration of tuberculosis bacteria has been described by Richards and Miller (1941). Patton and Metcalf (1943) have extended the work of Bock and Oesterlin (1939) to recommend the use of fluorescence for the detection of protozoan parasites in blood smears. Plate 1-c.

The use of the fluorescence microscope has also been described in the study of the metabolism of water-soluble vitamins in the American roach by Metcalf and Patton (1942) and by Metcalf (1943a). The same author (Metcalf, 1943b) has also described the fluorescence characteristics of a new biological pigment isolated from the gonads of fireflies.

Although phenomenal results have been reported from the application of fluorescence technics, the technical difficulties, both real and imaginary, which accompany such have limited wide acceptance. It is the purpose of this paper to point out simplifications in equipment and methods which can be made without reducing the quality of the results, and to enumerate and describe the auxiliary technics which can be used to supplement visual observations.

In prosecuting this study, the authors have taken great care to avoid recommendations which have not been thoroughly tested in our own laboratories. When simplifications of equipment and technics are indicated, explanation has been attempted on a sound theoretical basis.

Apparatus

The apparatus required for fluorescence microscopy is essentially the same as for exact microscopic work under the usual conditions. It consists of a source of ultraviolet radiations which should be mounted upon a simple optical bench to insure critical focusing, a system of filters to limit the wave-length range of the illuminating beam, a good monocular microscope, and an auxiliary filter to remove stray ultraviolet light which penetrates the microscope.

The Light Source. The principal innovation in the equipment is the source of light. In order to excite the emission of fluorescent light by the object according to Stoke's law, the exciting radiation

must be rich in rays of short wave length. The production of fluorescence in biological materials is excited effectively by rays between 350 mμ and 400 mμ. For this purpose the high pressure mercury vapor arc lamp appears to be the most satisfactory (Gage 1941). The G. E. types H-3 or H-4 have been used extensively. These can be installed in any good microscope-lamp housing. The type H-4 requires a special base. A ballast transformer is required for either lamp. These lamps have a life of 1000 hours, are readily obtainable, and can be purchased complete with housing for a sum not significantly greater than the cost of a good research model microscope lamp.

Other light sources which can be used for this type of work are described by Ellinger (1940b). These include the D. C. iron arc; the carbon arc; the magnesium arc; iron-salt-cored carbon arc; low pressure mercury vapor lamp; and for less critical work with strongly fluorescent substances, tungsten sources such as a 500-watt projection lamp (Graham 1942), a six-volt lamp burned at double rating or a photoflood lamp.

The lamp housing should have the same attributes for use with the fluorescence microscope as for ordinary work. It should be equipped with a secondary source such as a condenser; it should have a diaphragm; and it should be mounted on a rod or track so that its position relative to the microscope can be accurately adjusted. The latter point is important.

Filters. In order to observe the fluorescent light produced by the ultraviolet beam, all visible light must be removed by filters. The most suitable for fluorescence microscopy with biological objects is No. 584 (new No. 5840) red ultra which is obtainable from the Corning Glass Works. This filter transmits radiations between 300 mμ and 400 mμ, and a very small percentage of visible light. Filter No. 586 (5860), violet ultra, transmits almost no visible light, but has an ultraviolet transmission only about one half that of number 584. It is useful where an absolutely black background is desired without the use of dark-field. Ultraviolet transmitting filters numbers 587 (5874); 597 (5970), the red purple ultras: and number 585 (5850), blue purple ultra, transmit too much visible light to be desirable for many phases of fluorescence microscopy. This is also true of number 986, red-purple Corex A, which is recommended by Gage (1941) for ultraviolet microscopy. This filter has a very high ultraviolet transmission down to 250 mμ but passes so much visible light as to be undesirable for most biological work.

The earlier investigators have made use of glass or quartz absorption cells 1 or 2 cm. in thickness and containing a 1% solution of *p*-nitrosodimethylaniline to remove the blue, green, yellow, and orange rays. Blue "Uviol" glass filters may also be used in addition to this absorption cell to absorb blue and violet light not trapped by the *p*-nitrosodimethylaniline. This combination allows the passage of light from 280 mμ to 400 mμ.

With the mercury arc, a small amount of visible red light passes the red ultra filter number 584 and must be absorbed. This can be done by using a glass cell one centimeter in thickness containing a 5% to 10% solution of $CuSO_4$ to which a few drops of H_2SO_4 have been added. The solution should be renewed at intervals. A blue green Corning filter number 428 (4308), can be used in place of the $CuSO_4$ solution, but it is not as effective in removing the heat rays; and its concentration cannot be adjusted to give complete absorption of the red light. This is very important where red fluorescing substances are to be studied.

If a piece of opal glass of the same size as the ultraviolet filter is provided, a change from fluorescence observation to visible light is almost instantaneous.

Almost any good microscope can be used with a mercury lamp for fluorescence observations. Certain reservations do apply however, for the most satisfactory results.

Mirror. The usual plane microscope mirror is satisfactory for fluorescence work. The use of quartz prisms or the relatively inexpensive evaporated aluminum-front surface mirrors, or the aluminum disks supplied commercially is desirable but unnecessary, providing the intensity of the ultraviolet beam is sufficiently great. Where the intensity is low, the use of these mirrors is essential.

Substage Condenser. It is commonly stated that quartz lens systems in the condenser on the source and the substage condenser of the microscope are essential for fluorescence microscopy. (Gage, 1941, Ellinger, 1940b, and others). This equipment is expensive, and, as our investigations have shown, is unnecessary for the production of fluorescence in biological materials. This fluorescence is excited principally by rays with wave lengths between 350 mμ and 400 mμ, and in this region glass is sufficiently transparent to allow a high percentage of the activating rays to penetrate.

In selecting optical equipment it is important to use non-fluorescing lens systems for all parts.

Three types of condensers are commonly used in bright field microscopy. Of these, the aplanatic has given the most satisfactory

results under the conditions necessary for fluorescence. This condenser consists of three lenses one of which is aspheric. It provides for a greater concentration of light than the Abbé and gives a greater spherical correction. Usually the aplanatic condensers are separable to provide for use with lower power objectives. The Abbé condenser usually found on student and medical model microscopes is entirely satisfactory for fluorescence microscopy. There is no serious loss in intensity with this type of condenser over the aplanatic. Achromatic condensers may also be used, but due to their construction with 5 or 6 lenses, there is a loss of intensity brought about by the light absorption.

The condenser should be fitted with a removable dark field disk. Both visual and photographic observations are made with this disk in place. This gives a luminous object against a perfectly black background with most of the ultraviolet rays escaping the microscope objective. For visual work where great brilliance is required, especially with immersion lenses and very high magnifications, the darkfield disk may be removed.

A cardiod darkfield condenser was tried with the fluorescence miscroscope. Although the results with the ultraviolet illumination were good, they were not sufficiently superior to compensate for the inherent optical limitations of this type of condenser.

When objectives of 20× magnifications or higher are used, a drop of petrolatum or water should be placed upon the condenser to serve as a connecting fluid between condenser and slide. This is essential for high intensity illumination. With low power objectives, it is necessary to remove the top lens or lenses of the condenser in order to illuminate the field.

Slides. Ordinary glass microscope slides from 1.2 to 1.5 mm. in thickness may be used. It is important that the thickness be no greater than 1.5 mm. The quartz and Corex slides recommended by Ellinger (1940b) and Gage (1941) offer no special advantages when the intensity of ultraviolet light is sufficiently great.

Mounting Media. The usual mounting media contain brightly fluorescent components, and substitutes are necessary. For temporary mounts, petrolatum serves well. This also serves as a satisfactory immersion medium for use with oil immersion lenses. For permanent mounts, iso-butyl methacrylate (duPont) as recommended by O'Brian and Hance (1940) has proved superior to all other mounting media. It may be used with or without a cover glass, is water white, has a satisfactory refractive index, is readily soluble in xylene or toluene, and is very stable. It does not discolor with age.

Objectives. Achromatic, fluorite, or apochromatic objectives are satisfactory. For color correction, the apochromatic system should be superior to the achromatic system, but the additional lenses which comprise the apochromatic objectives reduce the intensity of the image to a noticeable extent. In general, the same rules apply to the use and selection of objectives for fluorescence work as for bright light microscopy.

Oculars. As with the objectives, the same rules apply in the selection of oculars as with ordinary microscopy. Either negative (Huygenian) oculars or positive (compensating) oculars may be used. High power oculars should be avoided. Best results are obtained with a 5× or 10× ocular.

Auxiliary filter. Stray ultraviolet light which penetrates the microscope can cause a disturbing fluorescence of the eye and will fog a photographic film unless an auxiliary filter is used. Several types of filters are available commercially; however, in our experience where color photography was practiced, a liquid filter consisting of a 5% solution of $NaNO_2$ contained in a plane-sided glass cell 5 to 10 mm. thick was the most satisfactory. This filter may be placed as a cap on the ocular providing the latter has a sufficiently high eyepoint, at the point of the diaphragm in the ocular, or at the point of the diaphragm in the tube of the microscope. The last placement is the most convenient.

Such filters as the Corning number 306 (3060) "Noviol" shade O, the Leitz filter number 8574a as recommended by Popper (1941), or the Zeiss Euphos filter are colored and appreciably alter the color of the image. These are optically superior to the liquid filter at high magnifications. A satisfactory yellow filter can be made by cementing yellow or amber cellophane between two circular cover glasses with isobutyl methacrylate. Gelatin filters can be used if the natural fluorescence of this material can be avoided.

Fluorescence Microscopy by Incident Light

Intravital fluorescence microscopy was introduced by Ellinger and Hirt (1930) and has been found useful for the study of spontaneously fluorescent materials in living organisms, and for the study of opaque tissues and organs.

Apparatus. The apparatus consists of an ultraviolet light source identical with that described previously, used in conjunction with a microscope equipped for incident illumination by a vertical illuminator. This may be one of two general types. An internal mirror or prism may be placed in the objective to direct the ultra-

violet light, which enters by a side opening, down through the lenses of the objective, which act as a condenser, to the object. There fluorescence is induced and the image may be seen through the microscope. In commercial types such as the Leitz "Ultrapak", the Zeiss "Epicondensor", and the Reichert "Epiluminator", the light is reflected by a centrally bored annular mirror or its equivalent

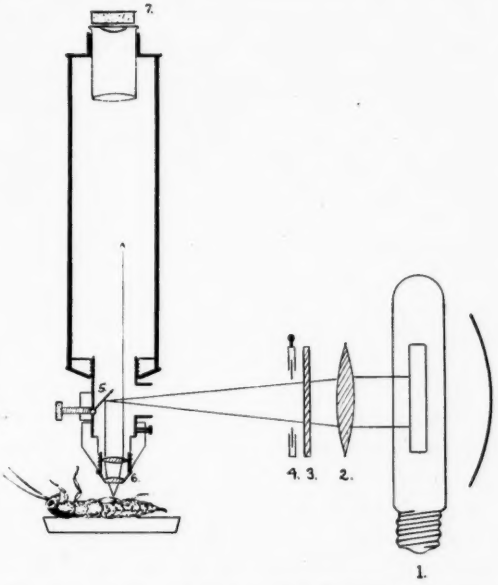


FIGURE 1. Equipment for Intravital Fluorescence Microscopy.

Key: 1. High pressure mercury vapor lamp
2. Condensor
3. Filter
4. Diaphragm
5. Front surface aluminum mirror
6. Divisible objective
7. Auxiliary filter.

through an independent condensing system which surrounds the objective. These devices have been used very successfully in intravital studies but are relatively uncommon in this country.

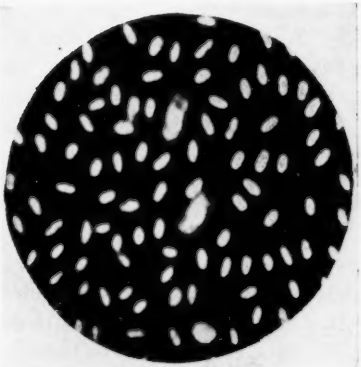
A very simple vertical illuminator can be built which will give excellent results in intravital studies by fluorescence. Our apparatus (Fig. 1) consists of divisible objectives from which the upper portion with the diaphragm has been removed. These are threaded

into a brass sleeve, which is threaded at its upper end with a standard objective screw. A circular opening drilled into the side of the sleeve allows the entrance of the ultraviolet light. Directly behind this opening is mounted a small front surfaced aluminum mirror which is fixed to a pivot which allows for focusing the light beam. The pivot is mounted upon a carriage controlled by a screw which effectively regulates the objective aperture, and serves as an internal objective diaphragm. Since in fluorescence microscopy the visible light source is in the object itself, there is no optical distortion due to oblique illumination. Although Ellinger (1940) states that the lenses of such an illuminator must be of quartz or of special ultraviolet transmitting glass, ordinary objectives may be used provided that the glass comprising the lenses or the cement used between the elements is non-fluorescent.

Only fine focusing is done in the conventional manner because of the fixed relationship necessary between the light source and vertical illuminator. The usual microscope stage is replaced by a stage capable of independent vertical adjustment, by which the object is brought into approximate focus. In use, the light source and vertical illuminator are aligned and the object is brought to the approximate focal point of the objective. The mirror of the illuminator is then tilted until the ultraviolet light beam is directed most intensely upon the object. This can be judged by the relative intensity of the fluorescence. By moving the mirror carriage the entire field can be evenly illuminated. When fluorescence is intense, a sharper image may be obtained by stopping down the diaphragm of the light source. With living material, continuous fine focusing is essential to give a three dimensional effect and to compensate for movements. Since a portion of the ultraviolet light is reflected back through the objective from the object, the use of an auxiliary filter is desirable. The $NaNO_2$ filter previously described is desirable for visual observations and essential for photography. Magnifications as high as 950× (with water immersion) have been used with excellent resolution. Plate 1-a. shows an intravital photomicrograph at 200× magnification.

For intravital work it is frequently necessary to perfuse the tissues with a saline solution. Ordinary physiological salines are comprised of solutions of electrolyte salts which are non-fluorescent and can be used freely. Ellinger and Hirt (1930) have used water immersion lenses irrigated with a moving current of saline to keep the lens clear. Dry 16 mm. and 8 mm. lenses give good definition even through a film of saline.

A. Intravital photomicrograph of the malpighian tubes of *Periplaneta americana*. From top to bottom: free riboflavin in the lumen of the tubes; a section of trachea; cytoplasmic riboflavin; and fat body. Magnification 200 ×. $NaNO_2$ auxiliary filter. Agfa Superpan Supreme.



B. Fixed cross section of larva of *Chironomus* sp. Stained with acridine yellow. Multicolored effects are obtained with a single stain. Magnification 200×. $NaNO_2$ auxiliary filter. Agfa Superpan Supreme.

C. *Haemoproteus* parasites in an avian blood smear. Stained with berberine sulfate. Magnification 900 ×. wratten G filter. Agfa Ultraspeed pan film.

PLATE 1

Photomicrographs by fluorescence of fresh, and fixed tissues; and of parasites in avian blood cells.

Histology by Fluorescence

The application of fluorescence technics to studies of microscopic anatomy and physiology demonstrates the distinct possibilities of these methods. Fluorescence finds its greatest application in the differentiation of functional tissues within an organism. In this application, the tissues appear in various colors depending upon their physiological state. Such studies can be made intravitally, with frozen or fixed sections, and with or without the use of fluorescent dyes. Some tissues contain natural fluorescent pigments which illuminate the tissues brightly when excited by ultraviolet radiations. These tissues may be plainly traced, and in the case of intravital studies, the physiological state may be estimated without the intervention of any foreign material. Naturally fluorescent materials frequently encountered include several flavin compounds (such as riboflavin), carotenes (vitamin A), chlorophyll, porphyrins, certain of the alkaloids, etc.

If natural fluorescence is not present or does not produce enough intensity for the study at hand, dyes may be added. The use of fluorescent dyes is of value in intravital work as well as with fixed tissues.

Fluorescent Dyes. Fluorescent dyes or fluorochromes (Haitinger 1938) include various coal tar dyes, and plant extracts such as certain alkaloids and plant pigments. They are applied to the tissues in a manner comparable to the use of usual histological stains. The coal tar dyes used can be classified into five groups. These include: xanthenes, acridines, thiazols, amino-azines, and azo dyestuffs. Specific dyes from these groups are listed in table 1 along with their properties. The principal dyes for fluorescence staining are italicized.

A most remarkable feature of these materials is their intense fluorescence at great dilutions. Most of them give a strong fluorescence color at dilutions of 1×10^{-10} and in the case of fluorescein, at a dilution of 1×10^{-14}. At such dilutions the daylight color of the strongest dyes is imperceptible.

Staining Procedure. The preparation of tissues for fluorescence staining is not significantly different than for usual histological technics. The tissue is fixed, dehydrated, infiltrated, sectioned, placed on ordinary microscope slides with a very thin film of albumin, stained, and mounted. The only differences are in the fixatives selected, the stains used, and the final mounting medium.

The salts of heavy metals, with the exception of zinc, halogen radicals, and nitro compounds, exert a quenching effect upon fluores-

cence. For this reason, fixatives which contain these must be avoided. The recommended fixative for most fluorescence work is 4% to 10% formalin. Dietrich's fluid (also known as Kahle's), which consists of a mixture of 30 cc. of 95% alcohol, 10 cc. formalin, 4 cc. glacial acetic acid and 60 cc. distilled water, has some advantages over plain formalin and can be used without effecting fluorescence. Haitinger (1938) recommends a fixative of 7 parts of 3% K_2CrO_4 solution, 2 parts formalin, and one part acetic acid.

TABLE 1.—IMPORTANT FLUOROCHROMES

Chemical Group	Fluorochrome	Color Index	Source*	Fluorescent Color	Use
Acridines	*Acridine orange*	788	N.A.	Orange-red	General
	Acridine yellow	785	N.A.	Yellow-green	General
	Acriflavine	790	Merck	Yellow	General, trypanosomes
	Coriphosphine O	787	Grb.	Yellow	General
	Phosphine 3R	793	Grb.	Yellow	Fat
	Rivanol		Metz	Green-yellow	Protozoan parasites
Xanthenes	Eosin Y	768	N.A.	Yellow	
	Fluorescein	766	N.A.	Green-yellow	Vital stain
	Rhodamine B	749	N.A.	Orange to red	Fat stain
Thiazoles	*Primulin*	812	E.A.	Blue-violet	General, virus and bacteria
	Thioflavine T. G. Ext.	816	E.A.	Light blue-green	General, virus and bacteria
	Titan yellow	813	Grb.	Blue	Fat
Quinone-imines	Magdala red, echt	857	Grb.	Red-orange	Fat
Phenyl methanes	Auramine O	655	N.A.	Yellow	Acid fast bacteria
	Acid fuchsin	692	N.A.	Red	Counterstain.
Azo dyes	Congo red	370	N.A.	Dull red	Counterstain.
	Thiazine red	225	Grb.	Violet	Fat
Alkaloids	Berberine sulfate		Merck	Yellow	Protozoan parasites (malaria)

*N.A.—National Aniline & Chemical Co. Metz—H. A. Metz Laboratory
Merck—Merck & Co. E.A.—Eimer and Amend
Grb.—Dr. G. Grubler & Co.

Almost all of the dyes mentioned are applied to the tissues from water solutions with dilutions ranging from 1/1000 to 1/10,000. This is near the range of saturation for many of the materials. At such dilutions, the staining time is from ½ minute to 5 minutes. The stains may be applied either by dropping the stain directly onto the sections or by dipping the slide as in usual procedures. After staining, the sections are washed in distilled water, dehydrated

through an alcohol series, cleared in xylene, and mounted in isobutyl methacrylate for a permanent mount or in petrolatum for a temporary preparation. Haitinger (1938) recommends a mounting medium consisting of 10 g. of clear, whitish gum arabic dissolved in 10 cc. of water and 5 cc. of glycerol with 1 g. of chloral hydrate added.

In intravital work the functions of living organs can be effectively studied by injecting fluorescent dyes into the organ to be studied or into the animal's blood stream. For such work the dyes employed must be water soluble, non-poisonous in the useful dilutions, non-diffusable in the living body, and must exhibit intense fluorescence in great dilution. The two most suitable dyes are the acid dyes, fluorescein or sodium fluoresceinate (uranine), and the basic dye, acriflavine hydrochloride. These are used as 0.1% to 1% solutions in physiological saline. Fluorescein is chiefly of use in the study of the function of organs. The color of its fluorescence varies with pH and consequently it is of use as an intravital hydrogen ion indicator. These color changes are easily visible at dilutions of 1/10,000,000. In dilute solutions the intensity of the fluorescence has a definite relation to the concentration of the dye. It thus serves as an indicator of the amount present. The relation of color to pH is described by Ellinger (1940a); pH is the only cause of change in color of this dye.

Acriflavine hydrochloride is used to differentiate cell structure. It is less intensely fluorescent than fluorescein and its fluorescence is partially quenched in basic solution so that it appears most readily in organs with an acid reaction.

Such dyes as rhodamine B, primulin yellow, thioflavine, berberine sulfate, vitamins A and G, Rivanol (2-ethoxy,-6,9,-diamino acridine lactate) and atebrine can also be used as intravital fluorescent stains. Rhodamine B reacts in a manner intermediate between fluorescein and acriflavine. The vitamins A and G can be used well as fluorescent dyes especially in studies of their metabolism. Such materials as Rivanol and atebrine can be studied and their therapeutic action estimated by their fluorescence reactions.

Tissue Differentiation. This phase of fluorescence microscopy is of especial interest to histologists, morphologists, and physiologists. The greatest advantage of the method is that the differentiation of tissue elements is varicolored without counterstaining. See plate 1-b. The differences in color produced by one stain may be explained by several factors.

The acridine and thiazol dyes are selectively absorbed by tissues,

and the fluorescence color produced varies greatly with the tissue to which the dye is fixed. This color differentiation can be partially explained, in the case of the acridine dyes by the fact that the fluorescence color is effected by pH. Since these materials also show differential absorption by various tissues, the two factors combine to produce a great variety of fluorescent colors. Dyes are taken up by various tissues at greatly different rates, and the contrast obtained is greatest when one tissue element is allowed to absorb a maximum amount without permitting any to be absorbed by the surrounding materials. The final fluorescence color visible to the eye is addative with the color of the natural fluorescence of the tissue being stained and the fluorescent color of the fluorochrome. The shade obtained depends upon the respective intensities of the two components.

As has been pointed out, a great variety of materials act as fluorochromes. The number actually needed for satisfactory permanent histological preparations can be limited to fewer than six. The acridine dyes, especially acridine yellow and coriphosphine O are the best general purpose stains. With either of them, most tissue sections exhibit very pleasing color differentiation. The thiazol dyes, thioflavine and primulin yellow, do not give as great color contrast as the acridines but give more intense fluorescence and are especially valuable for studies of musculature and structure. These are general purpose dyes. Special dyes and dye combinations may be used for specific purposes.

Although the best application at the present time for these technics appears to be the differentiation of tissues and organs rather than intracellular structure, cell structure and nuclear structure can be shown by special technics. Acridine yellow, acridine orange, coriphosphine O, phosphine 3R, and acriflavine all differentiate nuclei from cytoplasm clearly. This is also true of berberine sulfate. By staining with acridine yellow or coriphosphine O, and counterstaining with Congo red or acid fuchsin, a very distinct color differentiation of cytoplasm, nucleus, nucleolus, and chromatin can be obtained. This stain is applied by first staining for two minutes with Congo red and then two minutes with acridine yellow. The color differentiation is extremely sharp. Similar results can be produced with coriphosphine and fuchsin. A 2-to-1 mixture of these is effective after several minutes of staining. All of these dyes used in 0.1% solutions will give complete staining in one or two minutes.

Differentiation of Muscle. Almost all fluorochromes stain muscle

fibers. These appear light blue with acridine yellow, green with acriflavine, bright scarlet with acid fuchsin, blue with neutral red, and bright blue with primulin yellow. The most striking demonstration of muscle is produced by the diazotization procedure of Haitinger (1938), using the thiazol dyes, especially primulin yellow. The tissue is stained as usual with this dye and immediately after is washed in water and placed in a 2% aqueous solution of $NaNO_2$ to which has been added an equal volume of 10% HCl. The material remains in the bath about 15 minutes and is washed and treated for 15 seconds to a minute with a phenolic compound such as bromophenol, tetrabromophenol, resorcinol, or α-naphthol. By this procedure a polyazo dyestuff is developed in the tissue similar to the diazo dyes found in textiles. The fluorescence color produced depends upon the developing agent. For example primulin yellow diazotized with α-naphthol produces an incandescent yellow-white color in muscle tissue. The bromophenols give bluish colors and resorcinol, green. This same procedure can be followed with thioflavine and titan yellow and serves with other types of tissue as well as with muscle.

Differentiation of Nerve. Acridine yellow gives excellent differentiation of neuropile and cortical nerve tissue. The former appears light blue while the latter is light yellow to orange. With acridine orange the neuropile is blue gray and the cortical tissue orange brown. With acriflavine the neuropile appears blue and the cortical tissue yellow brown. Diazotization of primulin yellow gives good detail. The cortical tissue becomes dark violet to brown and the neuropile buff. Haitinger (1938) recommends diazotized thioflavine for nerve tissue.

Differentiation of Fat. Fat tissue in the fresh state has a natural blue to blue-violet fluorescence which may be confused with other blue fluorescent tissues. The fat can be differentiated by a variety of fluorochromes. The color produced varies with fixed and unfixed tissue and with the length of time in the fixative. This effect is generally a matter of a change in the shade of the fluorescent color. Popper (1941) has studied the distribution of vitamin A in fatty tissues and says that this material can be considered a vital stain for fat. He used a 1% aqueous methylene blue solution to stain fats for fluorescence examination. The staining requires 10 seconds. This causes the fat to fluoresce blue without obscuring the brighter vitamin A fluorescence. It is observable in visible light. Phosphine 3R, 1% aqueous solution, is a good fat stain. Three-minutes exposure produces a silvery white color. Popper (1941) says that

this stain differentiates more fats than can be seen with usual stains. He also used thioflavine S, 1% solution, which gave a violet fluorescence to the fat against a yellow background. This dye stained fewer lipids than phosphine 3R. The same author found magdala red or rose Bengal, used in 0.1% solutions and exposed for one minute, satisfactory. Haitinger (1938) recommends a freshly prepared alcoholic solution of chlorophyll which gives a fiery red fluorescence to fat, but this is impermanent. Several of the acridine dyes may also be used satisfactorily as fat stains.

Fluorescence Photomicrography

The image produced by the fluorescence microscope appears very bright and distinct by reason of the great contrasts, vivid colors, and the dark background; however, the light intensity is very low. No measurement of the intensity can be obtained with the usual photoelectric exposure meters. Exposure of photographic materials can be determined by selecting the optimum from a series of negatives exposed with the time of each exposure doubled. For photomicrography the dark-field disk and the auxiliary filter are always necessary in order to protect the emulsion from fogging by stray ultraviolet light.

From our experience, the exposure times for black and white film (Weston 50) using a 35 mm. camera with both the camera lens and the eyepiece in place ranges from 2 seconds to 5 minutes. The basic exposure for this type of condition is about 10 seconds.

When the durability of the preparation is not influenced by continuous ultraviolet radiation, [such as studies of vitamin A (Popper and Elsasser, 1941)], excellent results can be obtained by using Kodachrome (daylight). The exposure may be determined by calculation from a series of exposures made on black and white film or by a series of exposures made upon the Kodachrome itself. The selection of the method depends upon the requirements of the individual. Although color film gives excellent records and good illustrative material, the difficulties in duplicating exposure conditions make the value of the transparencies doubtful for standards of comparison for pH etc. Exposures of Kodachrome vary from 1–15 minutes with an average exposure time of about 2 minutes. Due to the low intensities, magnifications in excess of 500× are impractical. The microscope and camera must be mounted on a solid stand upon a vibrationless floor due to the long exposures.

Discussions of fluorescence photomicrography are to be found in Franke (1935), and Ellinger (1940).

Fluorescence Microspectroscopy

Positive identification of fluorescent materials in tissues can be made with a microspectroscope. It is possible to make spectroscopic measurements on extremely small areas of histological sections and isolated tissue elements.

The spectroscope equipment necessary may be either of the direct vision type employing Amici prisms or a grating type such as the Jelley microspectroscope (Jelley, 1936). In either case, calibration is simple since the mercury lines are easily observed by removing the filters from the light source.

Most fluorescent substances show an emission spectrum with a well defined maximum and these can be compared with a sample of the pure material suspected present by photographing the spectrum and comparing the negative densities. Fluorescence spectra of a number of biological materials have been determined and are to be found in the literature (Dhere 1939).

Conclusion

In the foregoing paper it has been the purpose to set forth the technics of fluorescence microscopy as they apply to biological research. The apparatus described has been thoroughly tested for many types of investigations over a period of three years and has proved satisfactory.

Although the fluorescence microscope as a research instrument has already made important contributions to the knowledge of biological phenomena, its possibilities for biological research are still limitless.

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PICRO-MALLORY

AN EASILY CONTROLLED REGRESSIVE TRICHROMIC STAINING METHOD

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ABSTRACT.—**Three modifications of Mallory's connective tissue stain are described and some features of the action of picric acid are discussed.**

In the first and most critical method the nuclei are stained in an iron hematoxylin and then differentiated in a picric acid solution containing orange G. This not only differentiates the nuclei, but stains all other elements yellow. The section is then washed in running water to remove the yellow color from all tissues except those which are to remain yellow in the final preparation (usually the erythrocytes). The section is next stained in an acid fuchsin mixture and then differentiated until the desired depth and contrast is obtained. Staining in anilin blue follows and this in turn is differentiated to suit. The section is then dehydrated and mounted.

In the second method the nuclei are stained in hemalum (e.g. Harris's) for a short time; the section is then rinsed and immersed in a mixture of picric acid and acid fuchsin and thereafter is differentiated; it is next passed into anilin blue w. s. and then differentiated and mounted as before. This is less critical than method I, but can be applied to large batches of slides at a time.

The third method is a one-solution method. After staining the nuclei in hemalum, the section is immersed in the "Picro-Mallory" solution, differentiated briefly, dehydrated and mounted. This modification, while being the least critical, is most suitable for routine use when the tissues have been fixed in a fluid containing chromate; the other commonly used fixatives, while giving useful results, are not so good.

This paper describes three modifications of Mallory's trichromic stain evolved from a previously published method (Lendrum and McFarlane, 1940); the name "Picro-Mallory" has been used for all the evolutionary stages of the stain. The most critical technic

[1]Part of this work was done while holding a Beit Memorial Fellowship.

(Method I) is applicable after all the commonly used fixatives, and it is of an easily controlled regressive character allowing the individual worker to emphasize the element in which he is most interested by varying the staining times. Up to the present, Method I (p. 35) has proved of great use in demonstrating the cytological changes in the pituitary (McLetchie, 1943), in showing many of the special features of spleen histology and in the investigation of the microscopical changes in the kidneys in nephritis. Apart from such highly critical aspects of the stain it has been possible by modification of the various solutions to evolve two additional less critical methods applicable to large batches of sections at the same time, the results so obtained being very good. In all cases the tissue elements stain as in Mallory's connective tissue stain.

In Mallory's method the erythrocytes are yellow, the cytoplasmic elements red (with certain exceptions) and the connective tissue blue. This technic has proved uncertain in the hands of many workers and further suffers from the inherent drawbacks that it is not under control and it is applicable only after chromate-containing fixatives or after suitable mordanting. Many modifications have been published since the original technic was described; of these Masson's is probably the best known and gives constant results after most fixatives. This stain is largely progressive in nature and has a further disadvantage in that all the acidophilic elements are colored red. Lendrum and McFarlane (1940) were able to get the classical colorations of Mallory's method by treating the section, after nuclear staining, in an alcoholic solution of picric acid containing orange G, and then following Masson's technic. This modification, however, did not increase control over the density of the dyes nor did it enable emphasis to be placed on one element in preference to another to any great extent. Much work has been done to overcome these difficulties and as a result two solutions have been evolved, one of which differentiates the red elements in an easily controllable manner, while the other removes excess of anilin blue from the cytoplasmic elements.

In the search for a "red differentiator", many substances and various combinations of substances were tried out. Alcohol in varying concentrations was found to remove the fuchsin with a rapidity increasing as the concentration of the alcohol increased; this method is not suitable as it does not act very rapidly and the action is non-specific, i.e. it removes the red from all elements at about the same rate. Alcoholic solutions of phosphotungstic and phosphomolybdic acid showed a marked improvement over the

alcohol, as the red dye, in this instance, was removed first from the connective tissue and then from the cytoplasmic elements. This action was not sufficiently strong on sections of tissue fixed in chromate solutions and further tended to remove the yellow color from the erythrocytes. Such solutions if used in Masson's technic in place of the aqueous solution of phosphomolybdic acid allow of much greater control. Alcoholic solutions of picric acid remove the red dye with a rapidity increasing with the concentrations of the two substances, and when saturated solutions of picric acid in absolute alcohol are used, the dye is removed very rapidly. Such a solution, while acting as a true differentiator, does so in the opposite manner from that desired, i.e. the red dye is removed first from the cytoplasmic elements and then from the connective tissue. In this way a Van Gieson effect is obtained. However, alcoholic solutions of picric acid and phosphotungstic acid were found to have the requisite action when the strengths of the various substances were properly adjusted. The strength of the differentiating action is dependant on the concentration of alcohol and of picric acid. Absolute alcohol containing 40 g. per 100 ml. phosphotungstic acid (i.e. approximately saturated) and 4 g. picric acid (PTA: PA = 1: 10) is the strongest differentiator. A ratio of about 10:1 is necessary if a Van Gieson effect is to be avoided. By diluting such a differentiator the rate of differentiation can be adjusted to suit the individual worker and the fixative in use.

Another disadvantage often encountered in the various Mallory modifications is that after staining in anilin blue the red elements have taken up some of this dye and have become degraded to a purple. In order to correct this a "blue differentiator" was evolved. It contains the same substances as the red differentiator in different proportions. It was found that alcohol in its lower concentrations removed the blue dye in a non-specific manner and at the same time removed the yellow color from the erythrocytes. The addition of phosphotungstic acid rendered the action specific; the addition of picric acid retained the color in the erythrocytes. This solution makes it possible to stain the connective tissue adequately and then to remove the blue dye which has overlayed the red of the cytoplasmic elements.

Before outlining the technic the action of picric acid will be discussed as it presents many interesting features of possible use to the specialist.

In Method I, after sections have been stained in the nuclear stain, they are immersed in saturated 80% alcoholic picric acid containing

0.25% of orange G. This differentiates the nuclei and at the same time stains all the elements yellow. When the section is washed in tap water, the dyes are washed out of the various elements at a rate depending on their affinity for the picric acid. If a section is observed at intervals throughout the process of washing, the yellow color will be seen first to leave the connective tissue, after which it comes out of the cytoplasm, although certain granular contents may hold it for a longer period. Fibrin resists decolorization for a still longer time, and erythrocytes are the last to be differentiated, being the most picrophilic. By controlling the washing time, it is thus possible to remove the picro-orange almost completely from all elements, except from the erythrocytes, these being left a robust yellow. If at this stage the section is passed into a solution of acid fuchsin, all elements with the exception of the yellow erythrocytes will rapidly take up the red dye. The amount of picric acid in the erythrocytes is sufficient to deviate the red from these cells. However, if the sections are left for a sufficient time in the red dye, the picric acid will be removed and the acid fuchsin will then color the blood corpuscles. The greater the amount of picric acid in elements other than the erythrocytes the longer these elements will resist staining with the red dye. Most frequently the deviating action of the picric acid is used to retain the yellow color in the erythrocytes and so to differentiate the red cells from such elements as fibrin; the specialist may, however, use the action in other ways by controlling either the differentiation of the picric acid during the washing or by carefully watching the section while in acid fuchsin, so that elements normally possessing similar tinctorial characteristics may be differentiated. In general it may be said that all tissues which are picrophilic have a similar affinity for acid fuchsin and further that acid fuchsin is deviated for a time from elements containing picric acid; this effect is directly proportional to their content of the acid.

During the investigation into the action of picric acid, Van Gieson's mixture was examined with the following interesting findings. If saturated aqueous picric acid is added, a few millilitres at a time, to a 1% solution of acid fuchsin, and sections are stained in the various solutions so obtained, the following sequence of events will be noted: When the proportion of picric acid to acid fuchsin has reached about 1:4 or 1:2 it will be seen that all the elements are a deep red with the exception of the erythrocytes, which are a brilliant yellow. As the proportion of picric acid increases, the cytoplasmic elements take the yellow, and the red dye does not stain so intensely in any

of the elements. As the proportions of the two substances approach those of the commonly used Van Gieson's modifications (picric acid to acid fuchsin 10:1), the picric acid gradually deviates the fuchsin from the acidophilic cytoplasmic structures and in the final Van Gieson only the connective tissue takes the red. Further increase in the picric acid content prevents the red dye from staining any of the elements. This principle is used in the routine "Picro-Mallory" method of staining large batches of slides when it is desired to avoid all unnecessary complications.

Solutions and Reagents

1. *Picro-orange.*

80% alcohol saturated with picric acid	100 ml.
Orange G. (C. I. 27)	0.25 g.

Sections can be left in this solution indefinitely without coming to harm.

2. *Acid fuchsin mixtures.*

Acid fuchsin (C. I. 692), Biebrich scarlet (C. I. 280) and ponceau 2R (C. I. 79) may be used as 1% solutions in 1% acetic acid. It has been found that a mixture of equal parts of 0.5% acid fuchsin and ponceau 2R gives a large variety of pleasant color tones suitable for use in method I.

Picro-acid-fuchsin (method II).

Picric acid	0.2 g.
Acid fuchsin	0.8 g.
2% acetic acid	100 ml.

3. *Phospho-picric differentiators.*

Stock solution

Phosphotungstic acid	25 g.
Picric acid	2.5 g.
95% alcohol	100 ml.

A. Red differentiators:

Red differentiator for the critical method (I)

Stock solution	40 ml.
95% alcohol	40 ml.
Water	20 ml.

This may prove too rapid in action for tissues fixed in some fixatives and for certain workers. Further dilution with water slowly decreases the rapidity of action and so the strength can be made to suit individual needs. In certain instances, e.g. when tissues have been left in chromate-containing solutions for a long time, decolorization is not sufficiently rapid; all that is necessary in these cases is to

transfer the section from the red differentiator into a saturated solution of picric acid in 80% alcohol, after which it is again rinsed in the red differentiator to re-mordant with phosphotungstic acid. Since the connective tissues will have been decolorized in the red differentiator to begin with, the action of the picric acid is to remove the red dye from the parenchyma as explained above (p. 31).

Red differentiator for the routine method

Stock solution	40 ml.
Water	60 ml.

This mixture is such that, in this department where tissues are fixed in Helly's fluid, differentiation is adequate in about five minutes. By increasing or decreasing the strength of the alcohol the action may be made more or less rapid as desired.

B. *Blue differentiators:*

Blue differentiator for the critical method (I)

Stock solution	20 ml.
Water	80 ml.

Blue differentiator for the routine methods (II & III)

Stock solution	10 ml.
Water	90 ml.

The strengths given will be found to be useful, but more rapid or slower action may be obtained by increasing or decreasing the concentrations respectively. The use of a blue differentiator for an adequate time cannot be over-emphasized. The resulting clarity and brilliance of the histological picture is largely dependant on the removal of all the excess anilin blue from the section.

4. *Anilin blue* (*C. I. 707*).

This solution is made up as advised by Masson, i.e.: add to 100 ml. boiling distilled water 2–3 g. anilin blue, boil for a few minutes and then add 2.5 ml. glacial acetic acid; allow the mixture to cool and then filter. Many different samples of anilin blue from different makers have been used from time to time and all have proved satisfactory. The acidity of this solution must be maintained and care must be taken not to carry over into the anilin blue any of the red differentiator.

5. *One solution Picro-Mallory.* (*Method III*)

Picric acid	0.2 g.
Acid fuchsin	1.0 g.
Anilin blue	2.0 g.
Phosphotungstic acid	1.0 g.
2% acetic acid	100.0 ml.

Preparation: To 100 ml. water add the dyes and phosphotungstic acid. Bring to a boil and allow to cool. Add 2 ml. glacial acetic acid and filter.

There are several points of importance with regard to this solution. The proportion of picric acid to acid fuchsin must be fairly constant. Similarly the amount of phosphotungstic acid present strongly influences the ability of the anilin blue to stain the connective tissues. The more acid present the more the blue is deviated from the tissues. The proportion of acid fuchsin to anilin blue is also important; if one is increased at the expense of the other, deficiencies and overlaying become apparent.

Procedures

Method I (critical)

1. Stain in Regaud's iron hematoxylin for the usual times.
2. Rinse in tap water and place the section in picro-orange until the section stains an even yellow and the nuclei are suitably differentiated; (3–5 minutes.)
3. Wash the sections in tap water until the yellow is removed from all elements with the exception of these which are to retain this color. In general only the erythrocytes will be left yellow. (The washing time decreases as the hardness of the water increases and in certain instances when the water is very hard it would be better to use distilled water for this process, in order to have the differentiation under control.)
4. Stain the section in the acid fuchsin mixture. Generally the erythrocytes are the only elements to be left yellow and, as long as they are not stained red, it is best to continue staining in the red dye as long as possible and then to differentiate strongly. (The staining time varies according to the tissue, fixative and the requirements of the individual worker, but will be found to be about 5–10 minutes.)
5. Rinse in 2% acetic acid and transfer to the red differentiator until most of the red has been removed and the various elements are suitably differentiated. (Again the time varies with the needs but will not usually be more than 5 minutes.)
6. Remove the differentiator thoroughly by rinsing in tap water (10 seconds) and then place the section in the anilin blue solution until the connective tissue and other elements are adequately stained. (Average time, 10 minutes; but this time varies with the above-mentioned factors.)
7. Rinse the section in 2% acetic acid and transfer to the blue differentiator for a sufficient time to remove the blue from the red staining elements and for excess to be removed from the connective tissues. (Time varies but should on no account be less than 1–2 minutes.)

8. Remove the differentiator by rinsing in 2% acetic acid; dehydrate rapidly and mount in a neutral balsam, e.g. the recently described synthetic resin balsams. Sections stained by this method and mounted in D.P.X. (Kirkpatrick and Lendrum, 1939, 1941) show no deterioration in twelve months, while sections stained by the original modification four years ago and mounted in this balsam are unchanged.

METHOD II (ROUTINE METHOD APPLICABLE TO LARGE NUMBERS OF SLIDES AT A TIME).

1. Stain in any of the ordinary hemalum solutions for the usual times. Harris' and Mayer's have proved to be useful.

2. Rinse in water and pass directly to picro-acid-fuchsin; (5 minutes frequently sufficient, but this may vary with the fixative used. Sections can be left in this solution indefinitely without coming to any harm, although they will require longer differentiation.)

3. Rinse in 2% acetic acid and place in the red differentiator; (5 minutes' time is the period for which the differentiator has been adjusted in this laboratory.)

4. Rinse in tap water to remove the differentiator and place the sections in anilin blue; (5–10 minutes sufficient.)

5. Rinse in 2% acetic acid and place in the blue differentiator. (The strength of this differentiator has again been adjusted so that adequate results are obtained in 5 minutes.)

6. Remove the differentiator in 2% acetic acid; dehydrate and mount as above.

METHOD III (RAPID ONE SOLUTION METHOD)

1. Stain in hemalum for the normal time.

2. Rinse in water and pass to the "Picro-Mallory" solution for a period depending on the fixative, tissue, etc. Five minutes in this solution gives a satisfactory picture; prolonged staining increases the density without any deterioration in the picture.

3. Rinse in 2% acetic acid and differentiate in the blue differentiator for about a minute. Remove the differentiator in 2% acetic acid.

4. Dehydrate rapidly and mount.

This method is obviously the least critical of the three, but it has the advantages that it is constant in action after fixation in most fixatives, although Zenker's or Helly's fluids are to be preferred. Large numbers of slides can be stained in a dish very rapidly; it

serves a useful purpose in differentiating the various tissues in much the same manner as Mallory's connective tissue stain, and finally from the nature of the stain the individual factor is more or less eliminated by the adoption of standard times.

Notes on the Methods

There is no doubt that the best results are obtained after fixation in solutions containing chromate, and of these fixatives Helly's has proved to be the best for a wide variety of tissues from both man and animals. All the commonly used fixatives, however, are suitable for methods I, II and III if the strengths of the differentiating solutions are adjusted to give the desired results.

All the solutions can be used in staining jars and if the proper precautions are taken remain active over long periods. The main points to be watched are: first, the acidity must be maintained in the anilin dyes, and second, thorough rinsing between the stages as indicated is necessary to keep the staining solutions pure.

The nuclei may seem to be rather faint after staining and differentiating in the picro orange. This is corrected when the sections are stained in the fuchsin which strongly re-inforces the existing nuclear stain.

My thanks are due to Dr. A. C. Lendrum for much helpful criticism and to Mr. W. Penny, Mr. W. Carson and Mr. J. Rennie for their technical assistance and judgment throughout the evolution of these methods.

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THE COMBINED GRAM-PAPPENHEIM STAIN AS MODIFIED FOR FILMS AND PARAFFIN SECTIONS

SARA A. SCUDDER, *Department of Pathology and Bacteriology, City Hospital, Welfare Island, Dept. of Hospitals, New York, N. Y.*

A combination of the Gram-Pappenheim stains for the examination of gonorrheal pus, cellular exudate and paraffin sections of formalin-fixed tissues has been described elsewhere (Scudder and Lisa, 1931). The crystal violet solution was made stable for the first time by employing phosphate buffers on the acid side of neutrality, and a stable counterstain was prepared for the first time from National Aniline dyes, ethylated methyl green and pyronin yellowish. Original findings were demonstrated by means of color plate I and II (Scudder, 1931) to show gonococci, pneumococci and cells in smears, and formalin-fixed tissue brought down to water in the usual way. A new color plate is published herewith to show the microscopic appearance of cells, Gram-positive and Gram-negative bacteria, higher bacteria, fungi and spermatozoa in the study of genito-urinary and gynecological cases. The method has a value in the field of medical jurisprudence. Crystals were well demonstrated, especially those resulting from sulfa drug therapy. The National Aniline methyl green batches numbered NG 10, 11, 13 to 19, and their batches of pyronin numbered NP 5 to 10 were found consistently stable. Earlier dyes were found either too purple or too blue for the technic and the most satisfactory dyes were found to require a ripening time of several days and could be prepared in amounts of from 1 to 4 liters and stored indefinitely without preservatives.

HISTORICAL COMMENTS

The first discriminating directions for buffering aqueous crystal violet solution by means of acid phosphates and for the selection of ethylated methyl green and pyronin yellowish dyes to be employed as a counterstain in the Gram technic were made by Scudder (1930–31). Laboratory research was begun in New York City in 1928–30 under auspices of the Biological Stain Commission and the Bellevue-Yorkville Health Demonstration, continued at the City Hospital, Welfare Island, New York City, and published as a monograph (Scudder, 1933) by the Medical Social Service Committee of Greater New York.

An alkaline Gram stain of temporary stability was first employed

in this country by Burke (1921) and the first suggestion of a universal method in which methyl greens and pyronins might be employed in the counterstain for the Gram technic was made by Pappenheim (1903). Sandiford (1937, 1938) published his method for a combined Gram-Pappenheim stain using European and British methyl greens, malachite green and pyronin bluish, calling attention to an earlier paper published by Fauth (1918) in which this combination had been advocated for the examination of smears from cases of chronic gonorrhea. The last three investigators employed alcohols, phenols, and glycerols as aids for dissolving and stabilizing the dye mixtures, but none of them differentiated between ethylated and methylated dyes, the percentage of dye content, the degree of solubility in water, or the "Blau" and "Gelb" designations of Grübler's dyes. Impurities such as free crystal violet or the presence of excess acid went unrecognized, as well as Pappenheim's earlier communication (1901) in which aqueous solutions of methyl green and pyronin had been employed.

The monograph by Scudder (1933) and other papers had established the fact that a new ethylated methyl green dye had been manufactured by the National Aniline Company and that pyronin yellowish was also being supplied by that company for use in the author's technic. These dyes had been found stable. Later research has shown that a dye content of about 76% is characteristic of the best methyl green (ethylated) dyes and that the purest dyes

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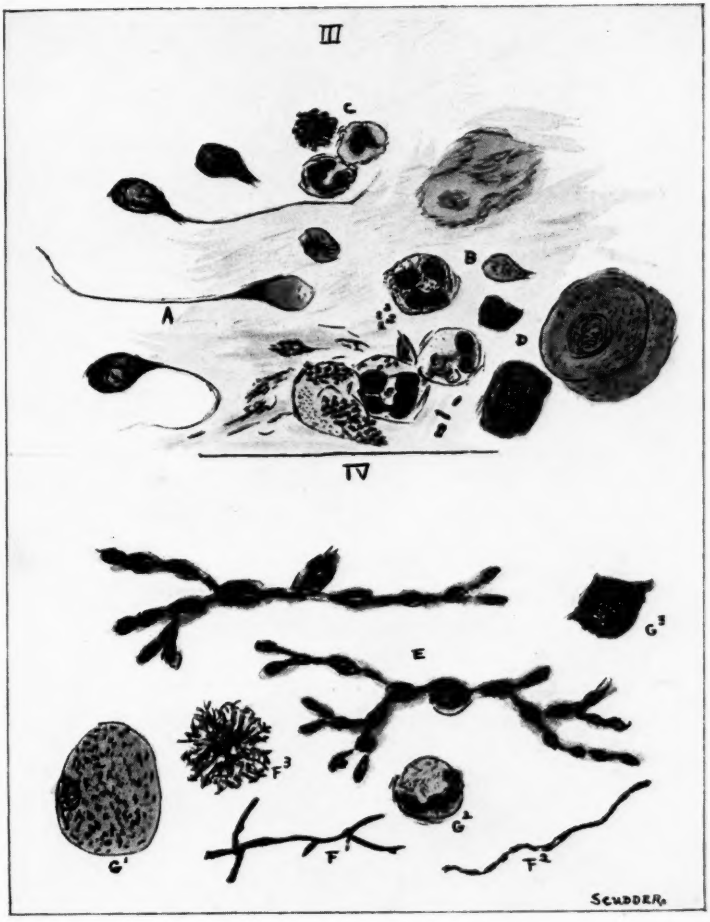
EXPLANATION OF COLOR PLATE[1]

3. Exudate from male cases (Gram differentiation)
   - A. Semen
   - B. Gonorrheal pus with disintegrated spermatozoa
   - C. Colony of gonococci
   - D. Mononuclear cells
4. Exudate from female cases (Gram differentiation)
   - E. Penicillia
   - F. 1 to 2—Actinomycetes culture
     3—Colony of Actinomycetes from axillary node of a guinea pig following inoculation with material from ovarian abscess in human case
   - G. 1–2–3—Different types of pus cells

[1]Plate 1 to 2 was published by Scudder (1931)
Gram differentiation:
1. Stain 3–5 minutes, 1% aqueous crystal violet buffered with acid phosphates, pH 6.6 to 6.9.
2. Flush 1½–2 minutes with alkaline iodine solution (Kopeloff and Beerman formula).
3. Decolorize rapidly, less than 10 seconds in pure technical acetone.
4. Counterstain 1½–2 minutes with National Aniline ethylated methyl green and pyronin yellowish solutions in hot distilled water (Scudder formula).


III
C
A
B
D
IV
E
G3
F3
G1
F1
G2
F2
SCUDDER.

require the longest dissolving and ripening process. Apparently Thomas (1936) had not seen the above publication when he wrote that the dyes employed by Scudder were unstable.

New Gram Stain with Ethylated Methyl Green and Pyronin Yellowish Counterstain

1. *Spread properly selected infectious material in thin films upon the slides, allow to dry, and fix by passing quickly through a flame several times.* Reagents and materials must be of highest purity; slides are chemically clean, dry and free from alcohol or acids. Merchurochrome, sulfapyridine and sulfanilamide are especially inhibitive to accurate staining; some newer drugs such as sulfadiazine do not seem to interfere. Glandular extracts, such as theelin, change the cytology, reduce the number of organisms and render cultures necessary for differential diagnosis.

2. *Flush the crystal violet solution upon the slide 3 to 5 minutes.* This solution is made up, not exceeding 1% in distilled water and stabilized by adding one part phosphate buffer (pH 6.9 or lower) in 10 parts of the crystal violet solution. Merck's Blue Label anhydrous Sorenson's phosphates are employed according to the method of Clark and Lubs. The dye mixture has remained stable for more than 10 years and one gallon amounts have been prepared at a time and stored in brown bottles in dark closets.

3. *Flush the slide with alkaline iodine solution for 1 to 2 minutes (Kopeloff and Beerman, 1922) after decanting the crystal violet.*

4. *Decolorize the slide rapidly in pure technical acetone for 10 seconds.*

5. *Air-dry thoroughly (moisture interferes) and apply the counterstain 1 to 2 minutes.* The slides are washed thoroughly in water and dried preparatory to examination by means of oil immersion lens. The counterstain is prepared in proportion of 0.65 g. of ethylated methyl green and 0.1 g. of pyronin yellowish in 100 ml. of hot distilled water, allowed to age several days or weeks and tested with known controls of bacteria and cells before being employed in routine diagnosis. Where the purity and dye content (76–80% approximate for methyl green and 50% approximate for pyronin yellowish) is known, no modification of formula is necessary.

Summary and Conclusion

Differentiation of wide range is obtained with this new Gram-Pappenheim technic: Polymorphonuclear cells, monocytes, epithelial cells, red blood cells, Gram-positive and Gram-negative bacteria,

fungi, spermatozoa and crystals are clearly defined as shown in Plates 3 and 4 and previously reported by Scudder in 1938. Crystal morphology is especially clear and excess crystals from therapeutic agents may be detected in urine and blood *cultures*. The technic has a place in medicolegal cases. Careful selection of the dyes is necessary and newly prepared solutions should be tested on known controls of Neisserian pus and mixed Gram-positive and Gram-negative bacteria, before being employed in routine diagnosis. Alcohols, phenols, and glycerols are found unnecessary in the counterstain. This counterstain has given as good results when used alone as the best Pappenheim combinations. An alternate counterstain of 0.1% aqueous basic fuchsin may be employed for comparison especially when blood cultures are stained for differentiation of gonococci, meningococci and staphylococci. The reagent solutions may be stored, in 1–4 liter quantities, in cool dark closets.

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NOTES ON TECHNIC

Acetic Methylene Blue Counterstain in Staining Tissues for Acid-Fast Bacilli

When counterstaining tissue sections with methylene blue after a carbol-fuchsin stain for tubercle or lepra bacilli, it is often difficult to achieve a counterstain which is neither too dense nor too light. While alum hematoxylin may be used successfully, this does not stain plasma cells, mast cells, and other bacteria, as does methylene blue.

For a number of years we used a 1:500 methylene blue buffered to pH 3.5 with citric acid and Na_2HPO_4. This was quite successful, but was subject to the mold fermentation of citric acid in aqueous solutions and sometimes went bad.

Recently we tried out a 3-minute stain in 1% methylene blue in 0.5% aqueous acetic acid solution after the usual hot carbol fuchsin, acid alcohol sequence, and secured quite selective nuclear staining with light blue cytoplasm, yellowish orange-red corpuscles, and bright red acid-fast bacilli.

By adding 20% alcohol to the solvent, the growth of organisms may be prevented. However, solutions of methylene blue in 0.5% aqueous acetic acid are relatively stable. They polychrome slowly and slightly. One such two-year-old solution had a spectroscopic absorption maximum of 645 mμ, indicating alteration about as far as azure B.

With this strength (1%) of methylene blue, it is more economical to stain in a staining jar than on the slides. One jar of approximately 150 ml. was used to stain 300 slides over a period of about 10 weeks, with quite satisfactory results throughout.—R. D. Lillie, *Senior Surgeon, U. S. Public Health Service; Division of Pathology, National Institute of Health, Bethesda, Md.*

Orientation of Young Intra-Uterine Embryos for Sectioning

Very young embryos of neural plate and early somite stages of animals such as the dog, cat, rabbit, etc., require careful orientation for sectioning so that the plane of the series will be a favorable one for fruitful study. This orientation may be accomplished by the following procedure:

After the specimen has been hardened by the fixing fluid, carefully cut the muscle coat from the expanded portion of the uterus in which

the embryo lies. Dehydrate the specimen in alcohol and clear in cedar wood oil or some other suitable clearing oil that makes the tissues translucent. While in the oil examine the specimen under a binocular dissecting microscope. Pass a strong beam of light thru the specimen and turn it about with a camel's hair brush. The embryonic area can be distinguished as a denser line stretched across the uterine cavity in the manner of a plane cutting a segment from a sphere near its surface. Slice off the side of the uterine wall opposite the embryonic area with a razor blade and the exact axis of the embryo can be observed under the microscope and marked as the specimen is embedded in paraffin. This method eliminates the danger of mechanical injury and damage by diffusion currents.—MELVIN C. GODWIN, *Department of Histology and Anatomy, West Virginia University, Morgantown, W. Va.*

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

COWDRY, E. V. **Microscopic Technique in Biology and Medicine.** 6 x 9¼ in. 206 pp. Cloth. Williams and Wilkins Co., Baltimore, Md. $4.00.

The author of this book has adopted the encyclopedic method of presenting the subject. So far as the reviewer is aware, this is the first time this method of discussing microtechnic has been adopted since the large encyclopedias of Ehrlich and of Krause. Really, however, this new book is hardly to be compared with the earlier German works. The author has endeavored—far from presenting, in German style, all available knowledge on the subject—to give in concise form the information of most practical value to the worker in microtechnic. Because of the brief and concise method of presentation, the book is small enough to find its place either on the desk or the laboratory table—can, in fact, almost be carried in the pocket. It ought to prove distinctly useful.—*H. J. Conn.*

PHOTOMICROGRAPHY

BAKER, JOHN R. **Tube-length in photomicrography.** *J. Roy. Micr. Soc.*, **62**, 112–5. 1942.

When a microscope is focused visually and then a camera is applied without further adjustment of focus, the object (or part of the object) seen by the eye is not in focus on the ground glass of the camera. The method here recommended for bringing the image into focus on the ground glass applies to the photomicrography of objects mounted in balsam or other medium of similar refractive index, and covered by cover glasses of the thickness for which the objective used is corrected. It is recommended that, after visual focusing, further focusing for the camera be achieved simply by extending the draw-tube of the microscope. The amount of extension necessary depends wholly on the eyepiece, and once this amount is accurately determined for a particular eyepiece, the ground glass may be discarded. In using this method, it is essential that the camera extension be fixed, the microscope held in sockets, and the photomicrographic lamp clamped in position.—*C. E. Allen.*

MICROTECHNIC IN GENERAL

FLEMING, W. D. **Synthetic mounting medium of high refractive index.** *J. Roy. Micr. Soc.*, **63**, 34–7. 1943.

Mounting media whose refractive index is close to that of glass serve well for stained specimens. But for colorless specimens such as diatoms, visibility is enhanced if the refractive index of the medium is higher than that of the object. Directions are given for the preparation of a synthetic resin designated *Naphrax*, whose refractive index is 1.7–1.8. That of diatoms is approximately 1.4.

In a glass vessel of 1500–2000 ml. capacity, preferably provided with a motor-driven stirrer and arranged for chilling in cracked ice, 200 ml. formalin is poured over 200 g. naphthalene. To the chilled mixture is added, very slowly and with constant agitation, a well-chilled mixture of 600 ml. glacial acetic acid and 300 ml. concentrated H_2SO_4 (sp. gr. 1.84). The temperature is held below 10° C. for the first 6 hr. Stirring is important during this period. Then the temperature is allowed to rise naturally to room temperature during 6–12 hr., stirring being maintained at least intermittently. The container is next surrounded by a water bath and the temperature of the mixture is raised gradually to 60° C., at which point it is maintained with agitation until a sample of the yellow resin, removed and washed in water, is brittle at room temperature.

Purification (which is important) is accomplished by washing the mass of crude resin in several changes of hot water, kneading the mass in the water with a heavy glass rod, then washing in two changes of warm 5% Na_2CO_3.

Next it is chilled and ground to a fine powder in a chilled mortar, pieces of ice being mixed with the resin to keep it brittle. It is washed by decantation in cold 5% Na_2CO_3, then in ice water. The powder is dried on a filter and washed with several changes of cold 95% alcohol. It is further washed by kneading or stirring the mass with a heavy glass rod in several changes of boiling 95% alcohol, then in 1 or 2 changes of boiling absolute alcohol. The mass is chilled, powdered in a dry, chilled mortar, washed with absolute alcohol, dried in air, and dissolved in pure xylene, toluene, or benzene. Dilution with the solvent continues until no more precipitate develops on standing at a low temperature. To the thin solution a small amount of anhydrous Na_2CO_3 is added and the solution is allowed to stand at least 24 hr. in an ice box, with frequent shaking during the early part of the period. It is decanted and filtered through the finest filter paper available. Finally the solvent is evaporated at a temperature not above 100° C. until the resin is a moderately firm mass.

It is advisable to add 1% of a plasticizer (dibutyl phthalate is recommended) to the pure resin.—*C. E. Allen.*

JELLEY, EDWIN E. **A review of crystallographic microscopy.** *J. Roy. Micr. Soc.*, **62**, 93–102. 1942.

The author gives a brief history of the development of the polarizing microscope and its accessory apparatus as used in optical crystallography, with reference to petrography and chemical microscopy.—*C. E. Allen.*

WATSON, J. M. **The ripening of Ehrlich's hematoxylin.** *J. Roy. Micr. Soc.*, **63**, 20–5. 1943.

Three methods, each involving the addition of an oxidizing agent, have been devised which make it possible to use Ehrlich's hematoxylin immediately after its preparation. All gave results, in selectivity and contrast as nuclear stains, equal if not superior to the naturally ripened stain, and of much greater reliability and consistency. Formula 1 stained more rapidly than either of the others.

FORMULA 1: 0.1 g. $KMnO_4$ dissolved in 100 cc. of 2% aqueous ammonia alum; 100 ml. 2% solution of hematoxylin in absolute alcohol; 10 ml. glacial acetic acid; 100 ml. glycerin.

FORMULA 2: 100 ml. 2% aqueous ammonia alum; 100 ml. 2% hematoxylin in absolute alcohol; 100 ml. glycerin; 10 ml. glacial acetic acid; 1.3 g. chloramine T.

FORMULA 3: 100 ml. 2% aqueous ammonia alum; 100 ml. 2% hematoxylin in absolute alcohol; 100 ml. glycerin; 40 ml. glacial acetic acid; 2 g. barium peroxide.—*C. E. Allen.*

WICKS, L. F., and SUNTZEFF, V. **Glyoxal, a non-irritating aldehyde suggested as a substitute for formalin in histological fixations.** *Science*, **98**, 204. 1943.

Glyoxal, or oxalic aldehyde, has been found to be a satisfactory substitute for formalin. A 2% glyoxal solution is equivalent to 10% formalin (4% formaldehyde) in fixing small pieces of tissue. Up to 10% glyoxal may be used for large masses of tissue, such as an entire human brain.

Glyoxal is available as a 30% to 40% aqueous solution, impure and too acidic for immediate use. It should be diluted to 10% and $CaCO_3$ added until effervescence stops, then filtered by suction through a rapid crepe paper. The solution will be faintly acid to litmus and should remain so.

The fixing solution has only a weak odor and is non-irritating. Results on tissues stained with hematoxylin-eosin are indistinguishable from those obtained with formalin fixed tissues.—*T. M. McMillion.*

DYES AND THEIR BIOLOGICAL USES

COPLEY, A. L., and WHITNEY, D. V. **The standardization and assay of heparin by the toluidine blue and azure A reactions.** *J. Lab. & Clin. Med.*, **28**, 762–70. 1943.

In view of the increasing use of heparin, a direct chemical assay method is desirable. The two following methods are described.

Method I: Prepare a 1:1000 solution of toluidine blue. (The dye used was that produced by the National Aniline Chemical Co. and certified under the number NU-3 and its composition required 1.6949 g. per 1000 ml. to yield a

1:1000 dilution of pure dye.) Prepare also a 1:1000 solution of azure A (certified as NAz7) by dissolving 1.1494 g. in 1000 ml. of water. In a series of tubes place 1, 2, 3, 4 and 5 Murray-Best units of heparin (1 unit equals 0.01 g. of the crystalline Ba salt of heparin), and make each up to 5 ml. with water. Let stand 15 min. and centrifuge at 3600 r.p.m. for 15 min. Decant the supernatant liquid and compare in a Duboscq colorimeter with 6 ml. of a 1:6000 solution of toluidine blue or azure A. Compute the residual dye in the supernatant liquid and subtract from 1000 γ (the original dye concentration) to get the amount of dye reacting with the heparin present. Experimentation gave an average of 140 γ of either dye reacting with 1 unit of heparin. Since the heparin unit was considered rather low, it was decided to establish the unit of heparin as that amount which reacts with 150 γ of dye.

Method II, carried out as follows, seems more accurate than method I: Prepare a 1:2000 solution of either dye. To this solution add 0.05 ml. increments of heparin solution, with swirling, from a burette, until precipitation starts, then drop by drop until a distinct blue equal to about 1:40,000 of the dye is observed among the heparin floccules. It was demonstrated that 150 γ of dye precipitates 1 unit of heparin. The test is reproducible with an accuracy of 5%, and is suggested as a practicable method for assaying heparin.—*John. T. Myers.*

ROTHMAN, STEPHEN, and FLESCH, PETER. **Isolation of an iron pigment from human red hair.** *Proc. Soc. Exp. Biol. & Med.*, **53**, 134–5. 1943.

An amorphous brownish powder with a metallic sheen, insoluble in water and organic solvents but soluble in alkalis, was extracted from bright red human hair. The purplish pigment, the reversible color change with a change in pH, and the absorption spectrum suggest that this substance belongs in the group of complex phenolic iron compounds. The phenolic OH group may be attached to a heterocylic ring containing nitrogen.—*M. S. Marshall.*

MICROÖRGANISMS

BEAMER, P. R., and STOWELL, R. E. **The use of tertiary butyl alcohol in bacteriologic staining procedures.** *J. Lab. & Clin. Med.*, **28**, 1599–602. 1943.

Tertiary butyl alcohol may be substituted for ethyl alcohol in the Gram and Ziehl-Neelsen staining methods. The following technic is satisfactory: Apply crystal violet solution (2 g. crystal violet in 20 ml. of tertiary butyl alcohol) for 10 sec., wash in tap water, Gram's iodine solution 10 sec., wash in tap water, 80% tertiary butyl alcohol 60–90 sec., wash in tap water, counterstain with safranin. For the Ziehl-Neelsen technic for acid-fast bacilli, use 5% HNO_3 in 50–80% tertiary butyl alcohol for decolorization. Tertiary butyl alcohol is readily available and reasonably priced. It may be employed if the present reagents become unobtainable or prohibitive in price.—*John. T. Myers.*

BRAY, J., and KING, E. J. **The phosphatase reaction as an aid to the identification of micro-organisms using phenolphthalein phosphate as substrate.** *J. Path. & Bact.*, **55**, 315–20. 1943.

The incorporation of phenolphthalein phosphate (prepared by King, E. J., *J. Path. & Bact.*, **55**, 311–4, 1943) in a solid medium such as Dorset's egg, which minimizes the diffusion of color, was found to provide a striking means of demonstrating the action of bacterial phosphatases, especially when the plate cultures were exposed to ammonia vapor. *Streptococcus pyogenes* reacted much more strongly than did pneumococci; "Certain of the diphtheroids were easily distinguished from *C. diphtheriae* by the intensity of their reaction"; and other differences were observed among clostridia, intestinal bacteria, and species of *Hemophilus*. A striking color plate illustrates some of these differences.—*S. H. Hutner.*

CROSSMAN, GERMAIN, and LOEWENSTEIN, ERICH. **Demonstration of tubercle bacilli in tissue by fluorescence microscopy.** *J. Lab. & Clin. Med.*, **28**, 1349–54. 1943.

The following method is recommended: Fix tissue in 10% neutral formalin for 12–24 hr., dehydrate, embed in paraffin, and cut sections 6–10 μ thick. Remove the paraffin completely (it is fluorescent), pass through alcohol series to water and place in auramine (auramine O, National Aniline, 0.1 g., distilled

water 100 ml., U.S.P. liquid phenol 4 cc.), steam gently for 3–5 min. (room temperature 30 min., 37° incubator 15 min., or 55° incubator 10 min.), wash in several changes of distilled water and decolorize with alcohol containing 0.5–1.0% HCl till quite colorless (about 45 sec.). Wash in distilled water. Stain 30 sec. in 0.1% aqueous methylene blue (National Aniline, Certification No. 18). Rinse in distilled water, dehydrate, clear in xylene and mount in xylene-clarite. Use a microscope equipped with a mercury vapor lamp with an iris diaphraghm, an ultraviolet transmitting filter and a dark-field stop. Place non-fluorescent sandalwood oil or Shillaber's immersion oil between the slide and the condenser. Enclose 3 sides of the microscope with a shield to exclude extraneous light. An aluminumized mirror is preferable to a silvered one. Tubercle bacilli appear as yellow, thin, luminous, slightly curved rods on a dark background.—*John T. Myers.*

KNAYSI, G., and MUDD, S. **The internal structure of certain bacteria as revealed by the electron microscope—a contribution to the study of the bacterial nucleus.** *J. Bact.*, **45**, 349–59. 1943.

Treatment of bacteria with 0.02% aqueous $NaHCO_3$ solution for about 2 hr. before films were prepared for study in the electron microscope reduced the opacity of the cells without affecting that of the internal granules; 5 hr. in 2% by vol. HNO_3 in water was also effective but started to dissolve the granules; 5 hr. in 10% HCl in water left the bacteria completely opaque. Granules believed to be nuclear were demonstrated in many but not all the species studied. In some bacteria granules were hard to demonstrate in young cultures even tho they were quite distinct in older cultures.—*V. Kavanagh.*

KRAJIAN, A. **A new and rapid staining method for Gram positive and Gram negative organisms in frozen and paraffin sections.** *J. Lab. & Clin. Med.*, **28**, 1602–6. 1943.

The following technic is recommended: Prepare 7–10 μ, mounted frozen sections. Stain 3 min. with alkaline methylene blue (Loeffler). Wash in tap water and dehydrate rapidly with three applications of ethyl or isopropyl alcohol. Differentiate rapidly with creosote-xylene (1 part creosote and 2 parts xylene), agitating for 5–10 sec. Pour off and apply creosote-fuchsin (2.5 ml. of 6% alcoholic basic fuchsin added to 50 ml. of creosote-xylene), agitating 15–20 times, and changing the solution once. Blot and apply creosote-xylene until the excess of red color is gone. Blot, clear 2 min. in xylene, and mount in gum dammar. For paraffin sections deparaffinize with 2 applications of xylene and 2 applications of absolute ethyl or isopropyl alcohol, bring down to tap water and proceed as above. The method is simple, rapid and demonstrates all bacteria including altered forms of Gram positive organisms.—*John T. Myers.*

LEVIN, MAX. **The effect of concentration of dyes on differentiation of enteric bacteria on eosin-methylene blue agar.** *J. Bact.*, **45**, 471–5. 1943.

The original eosin-methylene-blue agars were made with Grübler dyes which had less dye content than have the American dyes now in use. This has resulted in a disproportionate increase in the amount of methylene blue so that the media have a blue tinge instead of the amber desired; the colonies of the Salmonella and typhoid-dysentery groups take an atypical blue stain. To avoid these difficulties, the ratio of eosin to methylene blue should be approximately 6 to 1 and the pH of the medium should not exceed 7.4. These conditions are met by preparing the agar as follows: to 100 ml. of the melted basal medium (15 g. agar, 10 g. peptone, 2 g. K_2HPO_4 per liter) add 5 ml. of a 20% sterile solution of lactose, 2 ml. of a 2% aqueous solution of eosin-yellowish, and 2 ml. of a 0.325% aqueous solution of methylene blue (American dyes, sources not given).—*V. Kavanagh.*

McCLUNG, L. S. **On the staining of yeast spores.** *Science*, **98**, 159–60. 1943.

The author describes an improved method for staining yeast spores with malachite green. Solutions needed are: 1% malachite green dissolved without heating in 1% aqueous phenol; 0.5% aqueous safranin. The procedure is as follows: Prepare smear on a clean slide, air dry and fix lightly with a Bunsen flame. Cover entire slide with malachite green solution and steam 2 min. (do not boil) with a low Bunsen flame. Wash 1 min. in a gentle stream of tap-water; counterstain 30 sec. with aqueous safranin. Yeast spores should appear green, asci a deep pink.—*T. M. McMillion.*